

Annual Technical Report

Assessment of Regenerative Capacity in the Dolphin

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14. ABSTRACT Described herein is the technical information pertaining to Year 2 of a multi-year effort to determine and characterize the use of adipose (fat)-derived stem cells in the treatment of epidermal (skin) wounds. Adipose tissue was successfully harvested from the nuchal fat pad of six Atlantic Bottlenose dolphins via liposuction; cells released during the digestion of the adipose tissue were analyzed for cytology, assayed for the total number of colony-forming cells, expanded in culture, differentiated into multiple cell lineages and analyzed for stem cell surface markers. Cultured cells were also cryogenically frozen for future cell therapy treatment of dolphin skin wounds. Gene array analysis on the cultured cells show that a number of human related stem cell genes are positive. Furthermore, CD markers for known stem cell surface markers have been shown to bind to epitopes present on the cultured cells, lending further proof that the isolated cells are stem cells. Injections of stem cells into the skin wounds of dolphins display a more rapid healing than carrier solution alone.					
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a. Scientific and Technical Objectives

There is no information available on the distribution and functionality of adult stem cells in adipose tissue in the dolphin. However, it has been reported that cutaneous wounds in the dolphin heal very rapidly. We hypothesize that adult stem cells play an important role in wound healing in the dolphin. The objective of this proposal is to assess various adipose tissue depots in the dolphin for the presence of nucleated cells, to characterize those cells in order to establish their regenerative capacity and to use isolated, regenerative cells for treating wounds in a wound healing model in the dolphin. Vet-Stem will use its extensive knowledge and technical expertise to optimize the isolation of regenerative cell populations from dolphin adipose tissue, to characterize those cells with cross reactive CD markers and primers, to differentiate the cells into specific lineages and assess the therapeutic benefit of applying concentrated doses of the regenerative cells to wounds in a wound healing model. The knowledge gained from these studies will support the potential development of off-the-shelf cell-based “products” for treating a variety of pathologies and disease states in dolphins in the Navy and could extend knowledge to the treatment of sailors and other military personnel.

b. Approach

We propose to use techniques and protocols developed at Vet-Stem to characterize the nucleated cell preparations obtained from dolphin adipose tissue. The characterization will confirm the existence of adipose-derived stem cells (ASCs) in the dolphin, by demonstrating that a subset of cells from the isolated cell preparations are plastic-adherent in cell culture and these cells can be differentiated with specific media into adipogenic, chondrogenic, osteogenic and neurogenic cell lineages. This will provide the initial evidence of ASCs in dolphins. Further phenotypic characterization will be completed by assaying the cells for key surface proteins with existing immunological and molecular biological reagents for ASCs from other species. Positive identification of these existing reagents using cultured dolphin cells will prove that dolphins contain ASCs in their tissues. Cryopreserved cultured ASCs will be used as autologous cellular therapy for dolphin skin wounds. Finally, the cells will be tested for immunogenicity to develop an allogeneic (same species, universal donor cell line) model of cell therapy in the dolphin. After establishing these safe methods, we will use autologous and allogeneic ASCs to assess the therapeutic impact of applying a concentrated “dose” of regenerative cells in the wound healing model.

c. Concise Accomplishments

This Year 2 annual report builds upon the Year 1 annual report by continuing the culturing and the differentiation of the cells isolated from the digestion of dolphin adipose at multiple passages and thus providing further indication that these isolated cells can be termed stem cells. The cells were able to successfully differentiated into adipogenic (fat), chondrogenic (cartilage), neurogenic (neuronal), and osteogenic (bone) cell lineages at passages 2, 4 and 6. Freshly cultured cells from passage 4 were used for a gene array analysis using known human mesenchymal stem cell genes and a number of positive hits have been amplified; further confirmation of these genes is in progress. Phenotypic characterization of the cells using antibodies has resulted in the identification of numerous antibodies that can be used for characterization of the dolphin stem cells and include CD44, CD90, and CD105. These results indicate that the cells isolated from the dolphin adipose are adipose-derived stem cells based

upon the following set of criteria: cells are plastic-adherent, differentiate into multiple cell lineages, express gene sequences of known stem cell-related genes and express key surface proteins. Application of autologous cultured stem cells to skin wounds quickens the healing time.

d. Expanded Accomplishments

Characterization of Isolated Cells Cultured from the from the Digestion of Dolphin Adipose

The International Society for Cellular Therapy (ISCT) has established a set of minimal criteria for defining multipotent mesenchymal stem cells (Dominici M, et al., 2006):

1. Cells must adhere to plastic when placed in culture conditions;
2. Cells must express the Cluster of Differentiation (CD) markers CD73, CD90 and CD105 and lack expression of CD34, CD45, CD14, CD11b, CD79, CD19 and HLA-DR (major histocompatibility class II);
3. Cells must differentiate into adipocytes, chondroblasts and osteoblasts *in vitro*.

While these criteria are not universally accepted, they serve as the basis for the characterization of the cells isolated from the digestion of dolphin adipose. In the Year 1 annual report, it was established that these cells are plastic-adherent and are able to be expanded to multiple passages. Throughout the term of this contract, Vet-Stem has employed and utilized the latest techniques and information to ensure scientifically relevant results, in addition to those recommended by the ISCT.

Differentiation of Cells Cultured to Passage 6

In addition to showing evidence that the cells isolated and expanded from dolphin adipose are plastic-adherent, dolphin cultured cells expanded to passage 2, 4 and 6 have been successfully differentiated into multiple cell lineages as shown in Figures 1 through 4. The cultured cells respond to the respective induction media and have differentiated into adipogenic (fat; Figure 1), chondrogenic (cartilage; Figure 2), osteogenic (bone; Figure 3) and neurogenic (nerve; Figure 4) cells as shown.

Figure 1. Adipogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.

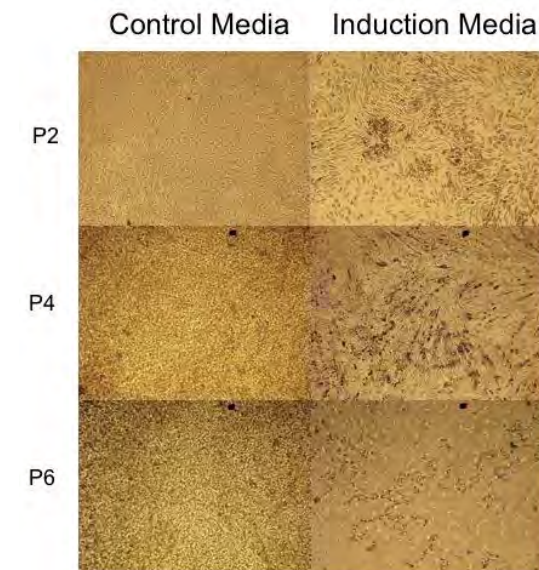
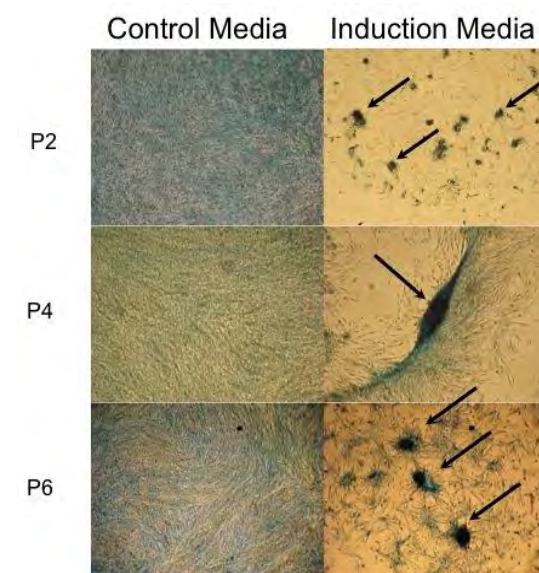


Figure 2. Chondrogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.



To briefly describe the procedure used in these differentiations, cells were grown to near confluence, trypsinized, seeded in culture plates and expanded to the required confluence. Induction media was then added to the wells, with fresh media added every 3-4 days. When the required time course was complete, cells were fixed and stained for protein residues and/or cell morphology specific to the respective differentiation.

The ability of these cells to differentiate at Passage 6 indicates that these cultured cells remain multipotent at later passage. Furthermore, an additional differentiation from those recommended by the ISCT has been achieved.

Characterization of the Cultured Cells through the use of Cell Surface Marker Analyses

To unequivocally determine that the cells isolated and cultured from the adipose of dolphins are in fact stem cells, fluorescent-assisted cell sorting (FACS) analysis was employed to identify key proteins present on the external surface of the cultured cells. Termed CD markers for Cluster of Differentiation, these surface markers have been previously referenced in literature to exhibit expression of these proteins on stem cells. As yet, there are no dolphin specific antibodies available and monoclonal antibodies targeted against human or canine epitopes were used. The analyses of these markers as they pertain to dolphin stem cells have focused around the more classical epitopes expressed on stem cells and those clearly and solidly identified in literature pertaining to stem cell related protein targets.

Figure 4. Neurogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.

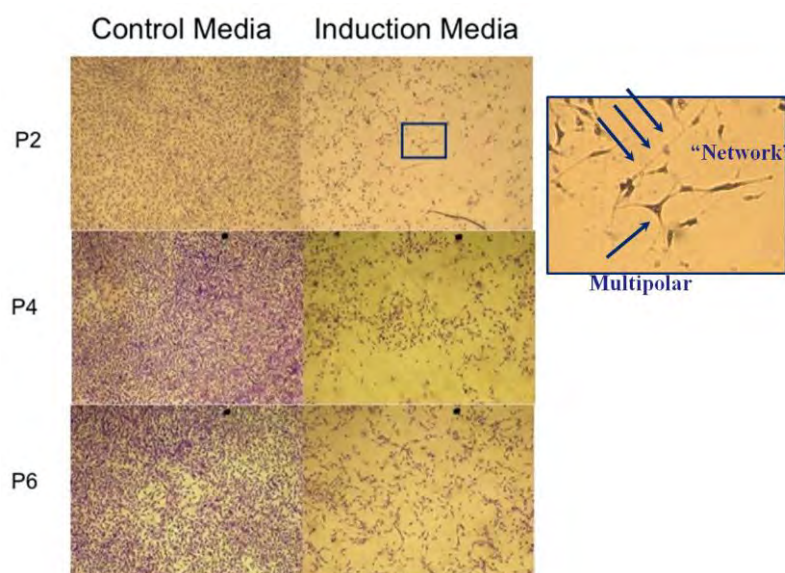
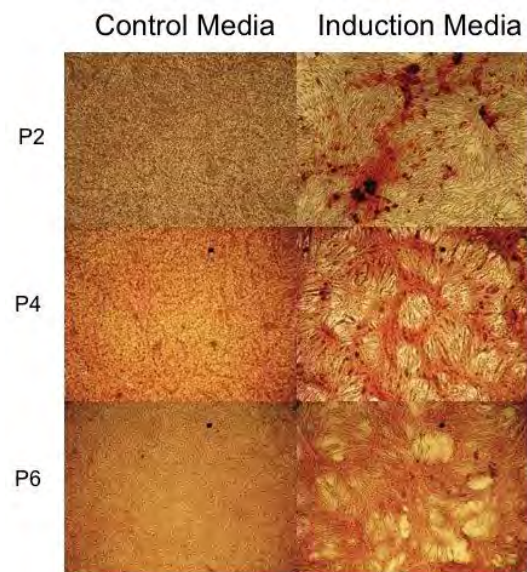
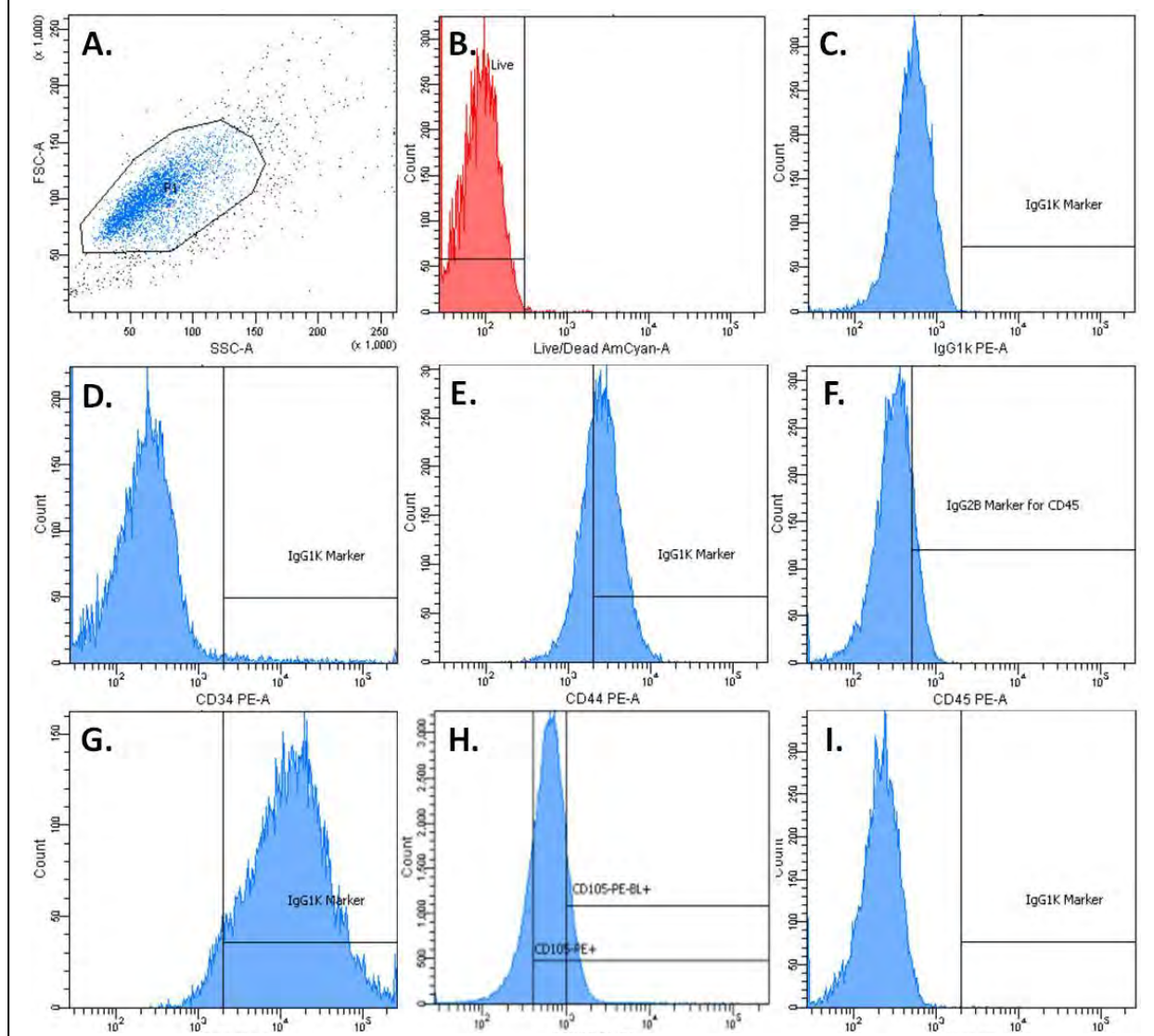


Figure 3. Osteogenic Differentiation of Passage 2 (P2), Passage 4 (P4), and Passage 6 (P6) Cultured Dolphin Cells.



To briefly explain the protocol used for analysis, previously cryopreserved cultured cells from were recovered and incubated with a LIVE/DEAD dye which is used to sort viable cells from dead cells. Following a series of washes and centrifugations, cells were then incubated with isotype controls or the selected antibodies and again washed and centrifuged prior to fixation in formalin.

Figure 5. Representative scatter plot and histograms obtained from the FACS analysis of passage 4 cultured cells from a single dolphin. A. Scatter plot of forward scatter versus side scatter. B. Gating on live cells only, excluding dead cells. Histograms for: C. Isotype Control; D. CD34; E. CD44; F. CD45; G. CD90; H. CD105; I. CD271.



The increased number of exposed amine residues on dead cells binds the LIVE/DEAD dye, making the dead cells highly fluorescent, as compared to live cells, which display a low level of fluorescent intensity. The dead cells were excluded from analysis and the live cells were analyzed for the specific antibody:fluorophore conjugate. Figure 5A-I shows a representative panel of analyses from a single dolphin at passage 4. The scatter plot in Figure 5A shows a population of relatively homogenous cells based on the forward scatter (size of the cell) versus side scatter (granularity). Throughout these analyses, the majority of the cells are viable, as shown in the gating at the top-left of Figure 5B; any cells excluded are deemed non-viable and are not analyzed for CD markers. Isotype controls were analyzed in order to set a background fluorescent threshold (Figure 5C) for CD marker expression in the dolphin cultured cells (Figure 5D-I). A similar pattern of expression was observed in 4 dolphin samples at passage 4 and in samples that were expanded to culture passage 6. The key proteins that show expression in

Table 1. List of antibodies that have been incubated with dolphin cultured cells.

Marker	Protein Name	Function	Antibody Raised Against	Bound to Dolphin Cultured Cells
CD29	Integrin Beta-1	Fibronectin receptor; recognition and cell adhesion	Human	-
CD34	Mucosialin	Cell-cell adhesion molecule	Canine	+/-
CD44	Hyaluran Cell Adhesion Molecule	Mediates cell adhesion and migration	Human	+
CD45	Leukocyte Adhesion Molecule	Protein tyrosine phosphatase; cell activation	Canine	+/-
CD73	Ecto-5'-nucleotidase	Catalyzes conversion of nucleotides	Human	+/-
			Human	-
			Human	-
CD90	Thy-1	Cell-cell interaction, adhesion and activation	Human	+
CD105	Endoglin	Cytoskeletal organization; cell morphology	Human	+
			Human	-
			Human	-
CD271	Low Affinity Nerve Growth Factor Receptor p75	Neurotrophin receptor; function not well defined	Human	-
			Human	-

dolphin cultured stem cells thus far are: CD44 (hyaluran cell adhesion molecule; Figure 5E) which is a receptor for hyaluronic acid and implicated in stem cell phenotypes; CD90 (thymocyte-1; Figure 5G) which mediates cell-cell interaction; and CD105 (endoglin; Figure 5H) which is involved in TGF-beta signaling. The lack of expression of CD34 (mucosialin; Figure 5D) which is a hematopoietic marker has been observed to have very low expression in all cultured dolphin samples analyzed thus far; an expected result.

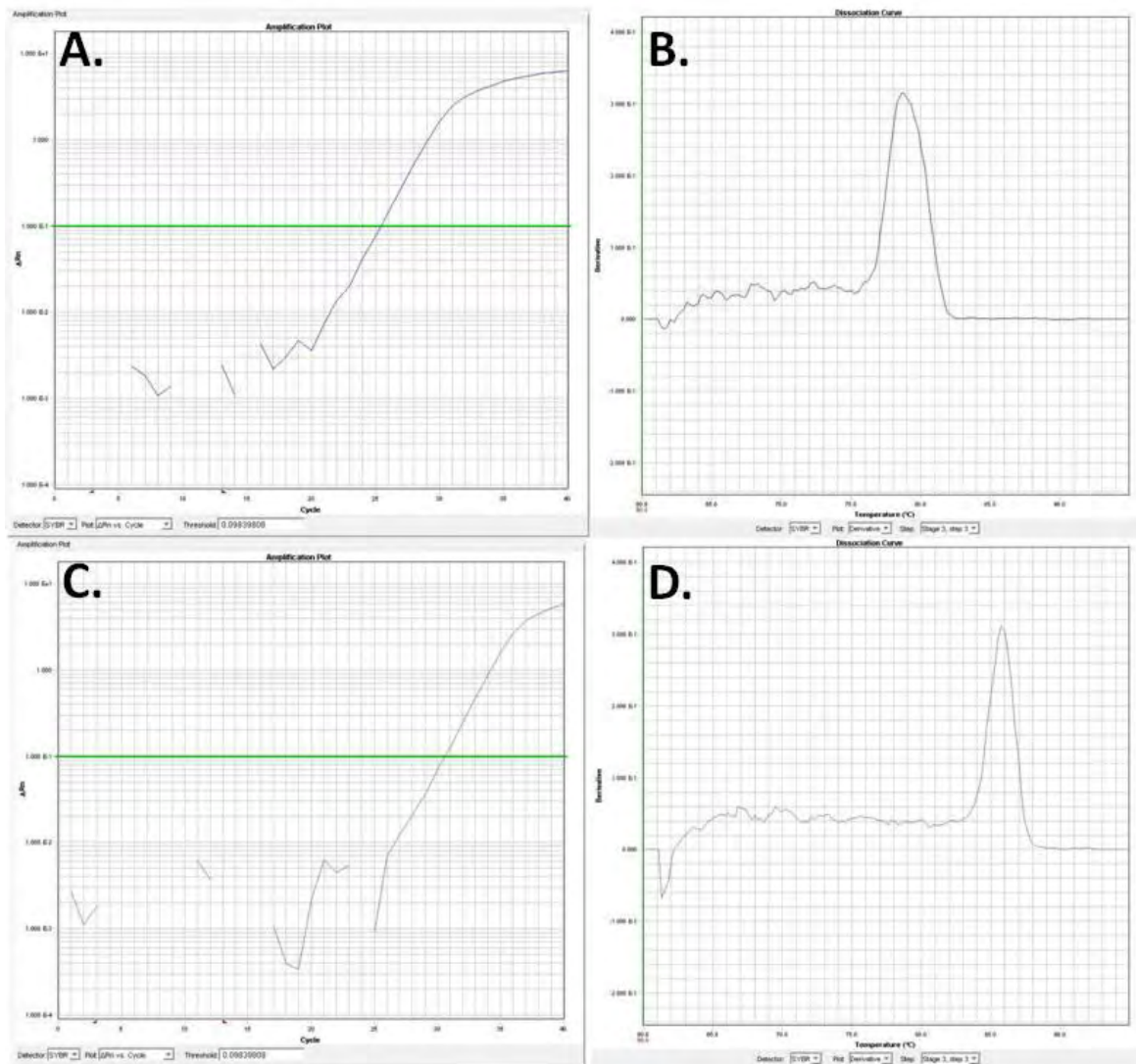
Table 1 shows a list of the antibodies used in these studies, including the CD marker name, the function of the protein, the species the antibody was raised against and whether the antibody bound to dolphin epitopes. The utility of the CD45 (expressed on leukocytes) antibody for use with dolphin cells will be confirmed by observing the expression of this protein in dolphin blood samples and will be performed concurrently with the mixed lymphocyte reactions (see e. Work Plan).

Characterization of the Cultured Cells through the use of Gene Arrays

To determine if stem cell related genes are expressed during the culture of stem cells, cells were freshly passaged and harvested for mRNA expression. In brief, the freshly harvested cells were pelleted and immediately frozen. Thawed cells were then immediately homogenized and placed on a RNA-purification column and the RNA isolated by a series of washes and centrifugation steps. Complementary DNA (cDNA) was generated and added to the real-time PCR array.

An array based on human mesenchymal stem cell genes was employed since there is little-to-no information pertaining to stem cell related genes in the dolphin. This real-time array makes use of fluorescence to monitor the live-time progression of amplification of the genes. As a gene is amplified, the fluorescence intensity increases, as seen in the amplification peaks in Figure 6A and C, which show the amplification of activated leukocyte cell adhesion molecule (ALCAM; CD166) and low-affinity nerve growth factor (LNGFR; CD271), respectively. To ensure that identified genes will have an optimal chance at positive correlation with sequences from dolphins, the melting curves were examined to better assess the specificity. If a single sequence

Figure 6. Representative plots of gene array data. Fluorescent amplification plots for A. Activated Leukocyte Cell Adhesion Molecule (ALCAM; CD166) and C. Low Affinity Nerve Growth Factor Receptor (LNGFR; CD271). Melting curve analysis for B. ALCAM and D. LNGFR.



has been amplified from the dolphin cDNA, then the melting curves will show a single peak in the melting curve analysis, as seen in Figure 6B (ALCAM) and Figure 6D (LNGFR).

Table 2 shows select genes related to stem cell proliferation. Sox2 is a transcription factor known to be implicated in maintaining pluripotency and along with Oct-4 expression is known to be a molecular indicator of embryonic stem cells. Transfection and expression of Oct-4 has also been shown to be a critical factor in the establishment of induced pluripotent stem cells. Sox9 is a transcription factor involved in chondrogenesis. While the exact function of LNGFR (CD271) is not known, antibodies raised against this marker have been used throughout the current literature to enrich adipose-derived stem cell populations. The expression of ALCAM and ICAM are both implicated in T cell activation, adherence and migration which stem cells are known to modulate.

Table 3 lists a number of genes pertaining to stem cell fate and pluripotency. Telomerase reverse transcriptase (TERT) maintains the length of the telomeres, thus maintaining pluripotency and inhibiting cellular senescence. Bone morphogenetic protein 4 (BMP4) has been shown to increase stem cell survival and maintain pluripotent characteristics. Both BMP4 and BMP7 have been implicated in cell fate, such as adipo-, chondro-, osteo- and myogenic cell lineages. TGF-beta is a promiscuous protein involved in maintaining pluripotency and cell fate across multiple lineages.

Table 2. Stem cell proliferation related genes identified by gene array analysis.

<i>Gene</i>	<i>Gene Name</i>	<i>Function</i>
SOX2*	SRY (sex determining region Y)-box 2	Stem cell related gene.
SOX9*	SRY (sex determining region Y)-box 9	Stem cell related gene.
NGFR*	Nerve growth factor receptor; CD271	MSC surface marker.
ALCAM*	Activated leukocyte cell adhesion molecule; CD166	MSC surface marker.
ICAM1*	Intercellular adhesion molecule 1; CD54	Integrin binding receptor.
ABCB1	ATP-binding cassette, B (MDR), member 1	Transport protein.
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	MSC proliferation and differentiation.
SMURF2	SMAD specific E3 ubiquitin protein ligase 2	MSC proliferation and differentiation.
VIM	Vimentin	Cytoskeletal marker for MSC.
FUT4	Fucosyltransferase 4	Blood group marker; present in undifferentiated MSCs.
NUDT6	Nudix (nucleoside diphosphate linked moiety X)-type motif 6	Putative proliferation gene.

Taken together, the ability for these cells to differentiate into multiple cell lineages, the expression of key CD markers and the positive expression of known stem cell related genes indicate that these cells isolated and cultured from the digestion of dolphin adipose are mesenchymal stem cells.

Table 3. Cell fate and pluripotency related genes identified by gene array analysis.

<i>Gene</i>	<i>Gene Name</i>	<i>Function</i>
TERT*	Telomerase reverse transcriptase	Maintain pluripotency.
BMP4*	Bone morphogenetic protein 4	Maintain pluripotency.
BMP7*	Bone morphogenetic protein 7	Maintain pluripotency; involved in cell fate.
TGFB1*	Transforming growth factor, beta 1	Maintain pluripotency; involved in cell fate.
EGF	Epidermal growth factor	Maintain pluripotency; involved in cell fate.
CTNNB1	beta-Catenin 1, 88kDa	Cell fate; Notch/Wnt signaling.
FGF2	basic Fibroblast growth factor 2	Maintain pluripotency; involved in cell fate.
MMP2	Matrix metalloproteinase 2 (gelatinase A, type IV collagenase)	Embryonic development, vascularization, and inflammation.
VWF	Von Willebrand factor	Maintain pluripotency; involved in cell fate.
PPARG	Peroxisome proliferator-activated receptor gamma	Maintain pluripotency; involved in cell fate.
HAT1	Histone acetyltransferase 1	Gene transcription.
JAG1	Jagged 1	Cell fate; Notch/Wnt signaling.
FZD9	Frizzled homolog 9; CD349	Cell fate; Notch/Wnt signaling.
ERBB2	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2; CD340	Her2; EGFR-like; cell maintenance.

Wound Healing Study

Dolphins are known to acquire skin wounds through numerous modalities including propeller strikes by watercraft, shark bites and “rake” injuries (termed for the characteristic pattern of lacerations) during the establishment of natural dominance within a group. To determine if stem cells could be used in the treatment of these wounds, a wound-healing model whereby a 10 cm long x 3 mm deep incision was made on each side of the midline, craniolateral to the dorsal fin was used. The wounds were irrigated with sterile PBS for a period of approximately 10 minutes using a procedure similar to those employed by Bruce-Allen and Geraci (1985). Injections containing either freshly recovered autologous cultured stem cells in 2 ml of media with 5% autologous serum (treated) or 2 ml of media with 5% autologous serum only (control carrier solution only – no cells) were injected into a single side along the entire wound axis. Personnel were blinded to which side received stem cells or carrier only solution. After a period of 10 minutes, the animal was then returned to their pen. Throughout the study, photographs of the wounds were taken and the health of the animal was continuously monitored by veterinarians and trainers.

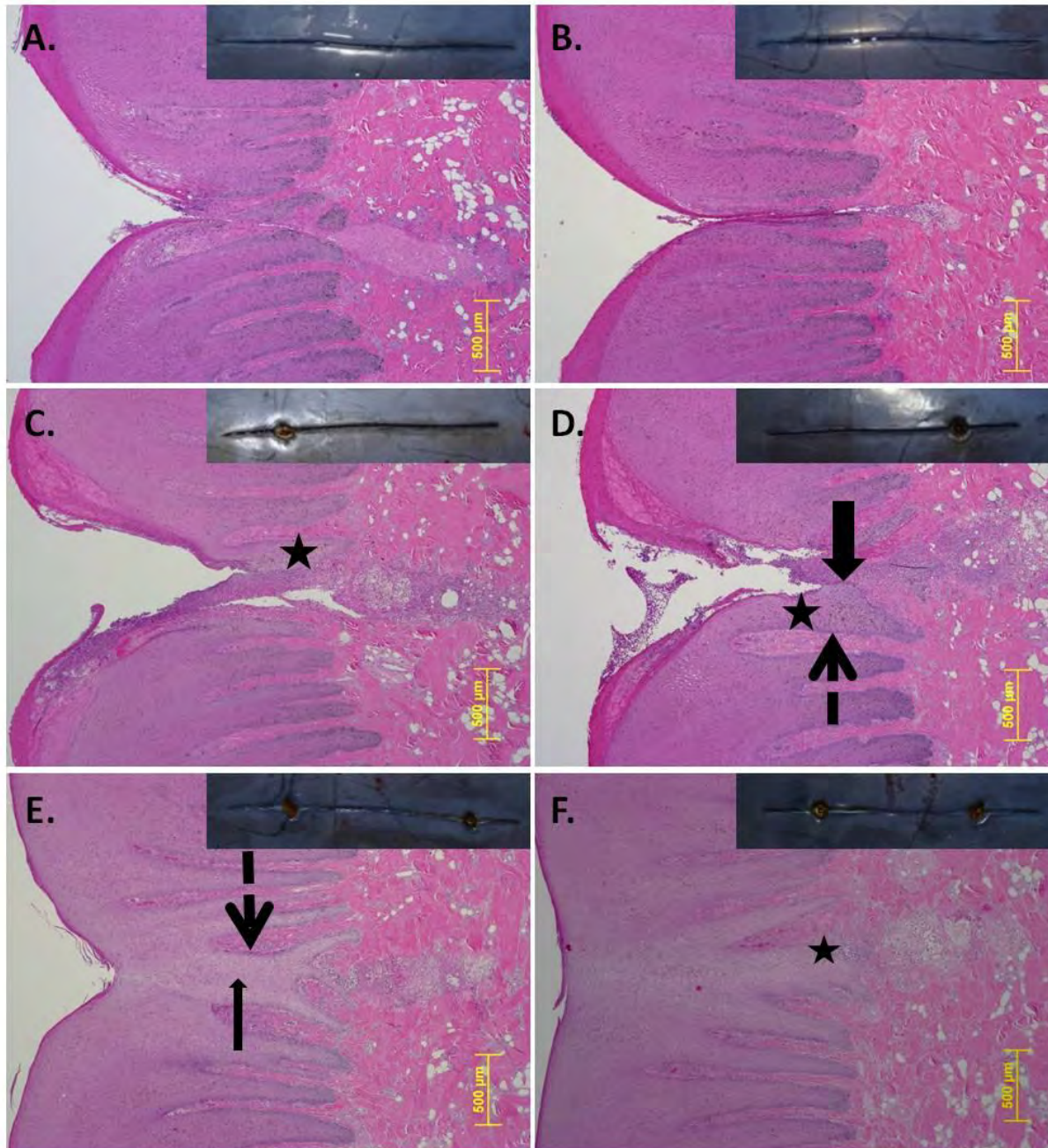
On Days 1, 5 and 15, an 8.0 mm circular biopsy, perpendicular to the wound axis, was taken, immediately placed in 10% formalin and labeled with the sample identity information. Prior to submission to the histopathologist, samples were transferred to pre-labeled 2 mL cryovials containing approximately 1 mL of fresh 10% neutral buffered saline. The biopsies were then embedded in paraffin, sectioned and stained with H&E (hematoxylin and eosin) and scored by a histopathologist who was also blinded to the injection status.

As shown in Figure 7, the left column displays the control treated side, injected only with media and 5% autologous serum (carrier solution only; A, C and E), whereas the right column shows the wound injected with stem cells and carrier solution (B, D and F). The first row of Figure 7 (A and B) shows the treatment at day 1, in which there were no qualitative or quantitative differences. By Day 5 (second row of Figure 7; C and D), a difference between the control and treated sides was noted by the histopathologist. The side treated with stem cells (Figure 7D) shows more inflammation and a greater number of mitoses suggesting more rapid cell division and thus, healing. Additionally it was noted that there also appeared to be a more established area of re-epithelialization present in the stem cell treated side, as compared to the control treatment (carrier solution only).

By Day 15 (third row of Figure 7, E and F), the epidermal layer is completely re-established in both control and treated sides. The number of mitoses is greater and the degree of dermal inflammation is lesser in the side treated with stem cells, indicative of a more rapid rate of healing (Figure 7F) as compared to the control treated side (carrier solution only; Figure 7E). Of particular note is the qualitative observation that the side treated with stem cells has a lesser surface depression than the side treated with carrier solution alone, displaying a significant difference in healing.

Throughout the procedure, which has since been repeated in a separate dolphin, both the NMMP personnel and the histologist noted that the sides treated with autologous stem cells are healing better and more rapidly than the sides treated with carrier solution only.

Figure 7. Histological examination of wound biopsies showing H&E stain along with a photograph of the wound prior to biopsy. Left Column is control treated (carrier solution alone – no cells); A, C, and E. Right column is the stem-cell treated; B, D, and F. Row 1 is Day 1; A and B. Row 2 is Day 5; C and D. Row 3 is Day 15; E and F. The yellow bars within each H&E stain are 500 microns across. In A and B there is no discernable difference. Stars in C and D show a subtle change between the control (C) and stem cell treated (D) sides where more epithelium has been established in the stem cell treated side, and is highlighted by the solid black arrow in D. The dashed arrow in D highlights a region proximal to the wound axis with more mitoses and rapid divisions than in C. Dashed arrow and solid arrow in E indicates a region with more inflammation and intra-dermal inflammatory cells, respectively. Black star in F indicates a region of the basal dermis with more mitoses than observed in the control injection, panel E.



References:

Dominici M; Le Blanc K; Mueller I; Slaper-Cortenbach I; Marini FC; Krause DS; Deans RJ; Keating A; Prockop DJ; Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular therapy position statement. *Cytotherapy*, (2006) Vol. 8, No. 4, pp. 315-7.

Bruce-Allen LJ and Geraci JR. Wound healing in the bottlenose dolphin (*Tursiops truncatus*). *Canadian Journal of Fisheries and Aquatic Sciences*, (1985) Vol. 42, pp. 216-228.

Catania JM and Harman RJ. Assessment of regenerative capacity in the dolphin. Defense Technical Information Center. Accession number: ADA530626.

e. Work Plan

We are currently in the process of verifying the nucleic acid analyses. Sequences identified from the gene array will be used for primer generation in quantitative PCR for positive identification of gene expression in dolphin. These primers will also be used to aid in the determination of the cellular fate of the stem cells in the wound healing model through the use of fluorescence *in situ* hybridization (FISH) analysis, whereby labeled nucleic acid sequences will be used in slides generated from the biopsies. Antibodies identified from the CD marker analysis will also be used to aid in the determination of cell fate on histological slides created during the wound healing studies. A third dolphin wound study is in the process of being performed and will be completed shortly.

The next phase of this project is to determine the safety of allogeneic cells. We have recently submitted an approved amendment of our IACUC protocol to increase the safety level associated with the mixed lymphocyte reaction (MLR) assay; a test for the immunogenicity of cells from a different donor performed *in vitro*. Originally, we proposed performing these studies in a xenogeneic model based on a human:dolphin MLR. However, to make a more thorough safety assessment and to better understand the data yielded by the MLR, we have now proposed to perform these studies using a dolphin:dolphin MLR. The results from these assays will determine how to proceed with our anticipated allogeneic model of cell therapy where stem cells cultured from one dolphin could be used in the treatment of another dolphin.

f. Major Problems/Issues

No major problem or issues have been encountered and Vet-Stem expects to complete project goals by the contract completion date.

g. Technology Transfer

This ONR-funded project has provided technology to Vet-Stem in some of the following internally-funded programs:

Clinical trials in allergic skin disease.

Further characterization of stem cell markers useful for equine and canine applications.

Vet-Stem is proceeding with the development of an advanced adipose collection device, partially as a result of the liposuction procedural difficulties encountered with dolphins. This device is currently being tested in equine and canine samples.

Additional commercial cases from animals with skin disease or diseases with related mechanisms of progression have benefited. These include renal, cardiac, inflammatory bowel syndrome and liver disease.

Vet-Stem has established pilot cooperative research and development agreements with Dr. John Morley of St. Louis University. This cooperative research agreement was a result of the 2010 Office of Naval Research Biosciences Program Review. An initial study of rodent adipose has been completed; the next phase will involve the use of cell therapy treatment to rodents with Alzheimer's disease.

Through interactions at the ONR Biosciences review program, there have been multiple fruitful interactions for studies that are in the process of being realized as potential treatments, such as epilepsy.

There are no plans for Technology Transfer of ONR-funded research and development in the upcoming year.

h. Foreign Collaboration and Supported Foreign Nationals

None; not applicable.

i. Productivity

Journal articles/book chapters/technical reports:

- a. "Adipose-derived stem cell collection and characterization in Bottlenose dolphins (*Tursiops truncatus*). SP Johnson, JM Catania, RJ Harman and ED Jensen. Submitted to *Stem Cell Research* September 2011.
- b. "Assessment of Regenerative Capacity in the Dolphin." Annual technical report. October 10, 2010. Defense Technical Information Center accession number ADA530626.

Workshops and Conferences:

- a. International Association for Aquatic Animal Medicine; Collection of Adipose-derived Stem Cells in Bottlenose Dolphins; Contributing Speaker: Shawn P. Johnson; Las Vegas, NV; May 12, 2011.
- b. ONR Marine Mammal Health/Pain/Antibiotics/Pain Program Review; Assessment of Regenerative Capacity in the Atlantic Bottlenose Dolphin; Invited Speaker: Jeffrey M. Catania; Arlington, VA; June 27, 2011.

Patents/Inventions:

- a. Not applicable.

Awards/Honors:

- a. Not applicable.

A second journal article is in the process of being drafted and will include the cellular characterization of the cultured stem cells, including CD Markers and RNA analyses. A third

paper will be submitted shortly after completion of the wound healing study. Plans are in development for the presentation of the cell characterization and wound healing at selected marine mammal scientific symposia during 2012.